

Physiochemical Characteristics of *Lactobacillus acidophilus* KH-1 Isolated from the Feces of a Breast-Fed Infant

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Abstract

Three lactobacillus strains, two from infant feces, and one from cow's milk, were selected among 172 isolates, from multiple sources, for further study based on the antimicrobial activities against six strains of pathogenic bacteria and identified as *Lactobacillus acidophilus*. The strains revealed a wide scope of spectrum against pathogenic bacteria. Viable *Lactobacillus acidophilus* KH-1 cell counts at pH 2.0 were slightly decreased to 1.42×10^7 CFU/mL from 4.18×10^7 CFU/mL, while remaining at 3.42×10^7 CFU/mL at pH 4.0 with the survival rate of 33.97% and 81.82%, respectively. At the concentration of 0.1% oxgall, *L. acidophilus* KH-1 kept growing up to 3.12×10^7 CFU/mL with a mean growth rate constant (k) of 0.25, and cell number was slightly decreased to 1.21×10^7 CFU/mL ($k=0.19$) with 0.3% oxgall, but remained at 7.6×10^6 CFU/mL ($k=0.17$) with 0.5% oxgall. *L. acidophilus* KH-1 had a D_{60} value of 7.14, with viable cell numbers 1.4×10^5 CFU/mL after heat treatment at 60°C for 30 minutes. Stability of *L. acidophilus* KH-1 at -20°C was significantly higher, when the strain was cultivated under the optimum growth temperature (54.41% and 54.35%) than at the temperature (13.53%).

Key words: *L. acidophilus* KH-1, survival rate, mean growth rate constant, D_{60} value

INTRODUCTION

The lactic acid bacteria (LAB) used in commercial starter cultures possess probiotic biochemical characteristics such as resistance to acidic environment, synthesis of antibacterial substances, production of exopolysaccharides, adherence to intestinal tract (1) as well as contributing to the flavour, texture and the nutritional value of the fermented products. Over the past decade, a new scope of research has been focused on the roles of the probiotics properties of lactic acid bacteria required for enhancement of the value of fermented dairy products (2-4). There are many important considerations for selecting supplementary lactic acid bacteria, including production cost of LAB starter cultures and the cost of production and maintenance of high number of cells in fermented dairy products. In addition, technical suitability, competitiveness, performance and functionality in production are well recognized selection criteria for the assessment of functional lactic acid bacteria (5,6). Production of antimicrobial substances such as bacteriocins, hydrogen peroxide, and organic acids and other inhibitory compounds are some of the important performances and functionalities of probiotics. The capabilities

for survival, proliferation, and metabolic activity in the gastrointestinal tract are also considered major requirements for competitiveness, as are resistance to bile and to acid. However, amenability to mass production and storage; adequate growth, recovery, concentration, freezing, dehydration, storage and distribution should be considered in relation to the technological suitability for commercial production of viable probiotics. It is almost impossible to assess the qualifications for use as probiotics on the basis of the phenotypic biochemical characteristics of the candidate lactic acid bacteria obtained from *in vitro* study. Currently, six species of lactobacillus; *L. acidophilus*, *L. casei* Shirota strain, *L. delbrueckii* subsp. *bulgaricus*, *L. johnsonii*, and *L. rhamnosus*, and five species of Bifidobacterium; *B. adolescentis*, *B. bifidum*, *B. breve*, *B. longum*, and *B. infantis*, and *Streptococcus thermophilus* are widely used in probiotic dairy products (7,8).

The purpose of this present study was firstly to isolate candidate lactic acid bacteria among the natural habitats of cow milk, goat milk, kimchi and infant feces. Secondly, to assess the appropriateness of *L. acidophilus* KH-1 isolated from the feces of breast-fed infants, as a probiotic bacteria by examining its technical suitability, performance and functionalities such as antimicrobial activity

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against pathogenic bacteria, tolerance to acidic environment, and resistance to the presence of oxgall, and survival rate over long storage periods at cold temperature.

MATERIALS AND METHODS

Lactic strains

Seventy four strains of LAB were randomly selected out of 172 total isolates from cow milk, goat milk, kimchi, and infant feces. Among these 12 lactobacillus strains were finally selected as candidate probiotic bacteria and 3 strains were characterized by their biochemical properties. *Lactobacillus acidophilus* KFRI 00151 used as a control strain was obtained from Korea Food Research Institute (Byundang, Korea). All the strains were maintained as lyophilized seed stocks in 16% skim milk mixed with 15% glycerol (Panreac, Badalona, Spain) and stored at -80°C for further studies. The lactobacillus strains were rehydrated in 10% skim milk and activated by incubating in lactobacillus MRS broth (Difco, USA) for 24 h at 37°C before using.

Plating of lactic strains for isolation

One milliliter samples were homogenized in 0.1% (w/v) sterilized peptone solution and diluted 10-fold in the same solution. Portions (100 μL) of appropriate dilution were spread onto MRS agar or MRS agar contained 0.02% sodium azide (Difco, USA). The plates were incubated anaerobically for 48 h at 37°C and colonies formed on the MRS agar were randomly selected.

Identification of lactic strains

On the basis of Gram reaction, morphology and catalase activity, growth at 45°C and at 5°C , isolates were selected and screened for the production of CO_2 from glucose and a colony of each isolate was identified by observing fermentation characteristics using the API system (La Balme-Les-Grottes, France) of API 50 CHL and with the API identification computer system (Bio Merium, France).

Viable cell counts

One milliliter of growth media was suspended into 0.1% peptone solution and 10-fold dilutions prepared by serial transfer into MRS. The number of colonies formed on MRS agar contained 0.02% sodium azide after cultivating at 37°C for 48 h was counted as total number of lactic acid bacteria.

Antimicrobial activity

Antimicrobial activity was assayed using the disc well diffusion method. The indicator pathogenic bacteria employed were *Staphylococcus aureus* ATCC 29213, *Listeria monocytogenes* ATCC 19113, *Escherichia coli*

ATCC 43894, *Escherichia coli* O157:H7 INK B00014, *Salmonella* Typhimurium KFRI 00251, and *Salmonella* Enteritidis ATCC 13096. These pathogens were purchased from Koran Food Research Institution (KFRI, Byundang, Korea). The challenged bacteria were grown in Nutrient broth (Difco, Detroit, USA) overnight and diluted to 10^8 CFU/mL with sterilized PBS; 100 μL of the cell dilutions were spread on the LB agar plates (Difco, USA). The spent culture supernatant (SCS) of lactobacilli grown in MRS broth for 48 hrs was obtained by centrifugation (5,000 rpm, for 5 min) and filtered through 0.22 μm sterile Millipore filters (Bedford, Massachusetts). Twenty microliters of the SCS adjusted to pH 5.5 with 1 N NaOH was dropped in triplicate onto the 8-mm diameter discs on the TSA agar. The agar plates were incubated at 37°C for 24 hrs for the six pathogenic bacteria. Diameters of inhibition zones were measured for evaluation of antimicrobial activity as no (-) inhibition <10 mm, good (+) inhibition >10 mm and strong (++) inhibition >15 mm clearance, respectively. Each assay was performed in triplicate.

Survival rate in acidic environments

MRS broths were adjusted to pH 2.0, and 4.0 with 0.1 N HCl. All the lactobacillus strains were inoculated at the level of 10^5 CFU/mL into each broth and maintained at 37°C for 2 hrs. The number of surviving cells was determined by counting the colonies formed on the MRS agar plate under anaerobic cultivation at 37°C for 48 hrs. The survival rate was expressed by the ratio of viable cell numbers after exposure for 2 hrs to acidic environments ($\log N_t$) to initial cell numbers ($\log N_0$).

Bile tolerance

The tolerances of *L. acidophilus* KH-1 and two other lactobacillus strains to bile, were evaluated in MRS broth cultures in which the lactobacillus cells were serially diluted in 0.9% (w/v) saline and 10^5 CFU/mL of each were inoculated into MRS broth containing 0.1%, 0.3%, 0.5% (w/v) of oxgall (Difco, USA). After anaerobic incubation at 37°C for 48 h, the colonies appeared on the MRS agar were counted. The equation for expressing the mean growth rate constant (k) was as follows:

$$k = \frac{\log N_t - \log N_0}{0.301 \times \text{incubation time}}$$

Heat shock at high temperature

The tolerance to heat shock of *L. acidophilus* KH-1 and two other lactobacillus strains was performed as follows; The viable cells (10^8 CFU/mL) of each lactobacillus strain cultivated in MRS broth was heated to 60°C in a water bath. After holding for 30 min, the broth was

taken out of the water bath and 1 mL plated onto the MRS agar plate. After anaerobic incubation at 37°C for 48 hrs, the colonies formed on the MRS agar were expressed as the survived cell number. An equation for the log reduction time (D₆₀) was as follows.

$$D_{60} = \frac{\log N_t - \log N_0}{\text{Holding time (min)}}$$

Freeze stability

To assess the stability at -20°C temperature, *L. acidophilus* KH-1 was incubated at different temperatures. The control was incubated at 37°C for 12 hrs and divided into two groups by further treatment; Group A was incubated further at 28°C for 24 hrs; group B was further incubated at 22°C for 24 hrs. Another two groups were incubated under different growth conditions, group C at 28°C for 36 hrs and group D at 22°C for 36 hrs without further treatments. All samples were stressed at -20°C for 24 hrs and then cultivated in fresh MRS broth at 37°C for 24 hrs to determine the revitalized cell number. The survival rate was expressed as the ratio of survived cell number (log N_t) after holding at -20°C temperature to initial cell number (log N₀) after cultivation at each growth temperature.

RESULTS AND DISCUSSION

Isolation of lactobacilli from different origins

Colonies of lactobacilli appearing on 0.02% sodium azide MRS agar were counted over 10⁵ CFU/mL from cow and goat milks, and over 10⁷ CFU/mL from kimchi and infant feces. A total of 12 lactobacillus strains was isolated from 4 sources; two strains from cow’s milk, two strains from goat milk, four strains from kimchi and four strains from infant feces. All these strains were found to be catalase negative, Gram positive rods, non-motile, negative for growth at 5°C and positive for growth at 45°C.

Identification of the isolates

After confirming morphological and biochemical properties of the isolate, three lactobacilli strains, two (KH-1 and KH-3) from infant feces and one (KM-2) from cow’s milk, were finally selected for further study based on the antimicrobial activities against pathogenic bacteria.

The result of fermentation patterns towards various carbohydrate sources by KH-1, KH-3 and KM-2 are described in Table 1. Bacterial identification was according to the API system (La Balme-Les-Grottes, France) of API 50 CHL. KH-1 was identified as *Lactobacillus acidophilus* 1 with a high similarity of 99.3% and

Table 1. Carbohydrate fermentation patterns of *L. acidophilus* KH-1

Carbohydrates	Reactions	Carbohydrates	Reactions
Control	-	Esculin	+
Glycerol	-	Salicin	+
Erythritol	-	Cellobiose	+
D-arabinose	-	Maltose	-
L-arabinose	-	Lactose	+
Ribose	-	Melibiose	-
D-xylose	-	Sucrose	+
L-xylose	-	Trehalose	-
Adonitol	-	Inulin	-
β-Methyl-D-xyloside	-	Melezitose	-
Galactose	+	Raffinose	-
Glucose	+	Starch	-
Fructose	+	Glycogen	-
Mannose	+	Xylitol	-
Sorbose	-	Gentiobiose	+
Rhamnose	-	D-Turanose	-
Dulcitol	-	D-Lyxose	-
Inositol	-	D-Tagatose	+
Mannitol	-	D-Fucose	-
Sorbitol	-	L-Fucose	-
α-Methyl-D-mannoside	-	D-Arabitol	-
α-Methyl-D-glucoside	-	L-Arabitol	-
N-Acethyl-glucosamine	+	Gluconate	-
Amygdalin	+	2-keto-gluconate	-
Arbutin	-	5-keto-gluconate	-

+: fermented; -: not fermented.

designated as *Lactobacillus acidophilus* KH-1. This species showed the same carbohydrate fermentation patterns as the isolate of Yu et al. (9) from piglet feces.

Antimicrobial activity of the isolates against pathogenic bacteria

To evaluate the antimicrobial activities of the three isolates, *Staphylococcus aureus* ATCC 29213, *Listeria monocytogenes* ATCC 19113, *Escherichia coli* ATCC 43894, *Escherichia coli* O157:H7 INK B00014, *Salmonella* Typhimurium KFRI 00251 and *Salmonella* Enteritidis ATCC 13096, were used as indicator pathogenic bacteria. In order to elucidate the antimicrobial activity, the pH of the supernatant of four lactobacillus strains were adjusted to pH 5.5 and soaked on discs implanted on TSA agar plates of the six pathogenic bacteria. The antimicrobial characteristics of the isolates and *L. acidophilus* KFRI 00150 are summarized in Table 2. The supernatant of the four lactobacillus strains tested showed antimicrobial activities against the pathogenic bacteria. *L. acidophilus* KH-1 made clear zones of 9 to 14 mm around the disc on four TSA agar plates of *L. monocytogenes* ATCC 19113, *E. coli* O157:H7 INK B00024, *S. Enteritidis* ATCC 13096, and *S. Typhimurium* KFRI 00251 and clear zones over 15 mm against *S. aureus* ATCC 29213 and *E. coli* ATCC 43894. *Lactobacillus* spp. KH-3 also made clear zones of 9 to 14 mm around the disc on three TSA agar plates of *E. coli* O157:H7, *S. aureus*, and *S. Typhimurium*. *Lactobacillus* spp. KM-2 made clear zones of 9 to 14 mm around the disc implanted on four TSA agar with *E. coli* O157:H INK B000247, *S. aureus* ATCC 29213, *E. coli* ATCC 43894, and *S. Typhimurium* KFRI 00251, while no clear zones were formed in TSA agar of *L. monocytogenes* ATCC

19113, and *S. Enteritidis* ATCC 13096. The other lactic strains showed weak antimicrobial activity against only two pathogenic bacteria.

In this study, although the pHs of the supernatants of the four lactobacillus strains were adjusted to pH 5.5, Tsai et al. (10) proved that some bacteriocin played their roles at low pH values. Therefore, we could suggest that a kind of bacteriocin from the isolates may possess antimicrobial activity.

Effects of acidic environments on the lactobacilli

The stabilities of lactobacillus cells obtained from either *in vivo* or *in vitro* study are similar according to Havennar et al. (2). Most lactobacilli grow more slowly at low pH, because of the loss of viability of the cells damaged at acidic conditions. However, the relative tolerance of lactobacillus to acidic environments is dependent on the strain of bacteria (11). The viable cells of the isolates were determined after 2 hrs incubation at 37°C in MRS broth after adjusting to pH 2.0 and pH 4.0. Table 3 showed that viabilities of lactic acid bacteria were significantly affected at pH 2.0. Viable cell of *Lactobacillus acidophilus* KH-1 at pH 2.0 was decreased to 1.42×10^7 CFU/mL from 4.18×10^7 CFU/mL, while those of KH-3 and KM-2 were decreased to 1.52×10^6 CFU/mL and 1.24×10^6 CFU/mL, respectively. However, all the lactobacilli at pH 4.0 remained more than 10^7 CFU/mL, with fair survival rates.

The survival rates of *L. acidophilus* KH-1 were 33.97% at pH 2.0 and 81.82% at pH 4.0 and *L. acidophilus* KFRI 00150 also had good viable cell numbers with a survival rate of 37.27% at pH 2.0 and with a survival rate of 71.74% at pH 4.0.

Yu et al. (9) insisted that *L. acidophilus* P1 and *L.*

Table 2. Antimicrobial effects of lactic acid bacteria against pathogenic bacteria

Strain	<i>L. monocytogenes</i>	<i>E. coli</i> O157:H7	<i>S. aureus</i>	<i>S. Enteritidis</i>	<i>E. coli</i>	<i>S. Typhimurium</i>
	ATCC 19113	INK B00014	ATCC 29213	ATCC 13096	ATCC 43894	KFRI 00251
KFRI 00150	+	-	++	-	++	-
KH1 ¹⁾	+	+	++	+	++	+
KH2	+	-	+	-	-	-
KH3	-	+	+	-	-	+
KH4	-	-	-	+	+	-
KM1 ²⁾	+	-	+	-	-	-
KM2	-	+	+	-	+	+
KG1 ³⁾	+	-	+	-	-	-
KG2	-	-	-	-	-	-
KC1 ⁴⁾	-	-	+	-	-	+
KC2	+	-	-	-	-	-
KC3	-	+	+	-	-	-
KC4	+	-	-	-	+	-

¹⁾KH means infant feces of Korean baby. ²⁾KM means kimchi. ³⁾KG means Korean goat milk. ⁴⁾KC means Korean cow's milk. -: no clear zone was formed around the disk; +: 9~14 mm of clear zone; ++: 15~25 mm of clear zone.

Table 3. Survival rates of lactobacilli in MRS broth adjusted to low pH (CFU/mL)

Strains	Initial viable counts	pH 2.0		pH 4.0	
		Viable counts	Survival (%)	Viable counts	Survival (%)
<i>L. acidophilus</i> KFRI 00150	3.22×10^7	1.20×10^7	37.27	2.31×10^7	71.74
<i>L. acidophilus</i> KH-1	4.18×10^7	1.42×10^7	33.97	3.42×10^7	81.82
<i>Lactobacillus</i> spp. KH-3	3.12×10^7	1.52×10^6	4.87	2.47×10^7	79.17
<i>Lactobacillus</i> spp. KM-2	1.94×10^7	1.24×10^6	6.39	1.40×10^7	72.16

acidophilus P2 have retained their viable cell numbers (10^7 CFU/mL) of inoculation without rapid loss of viability after incubation at pH 2.0. Kim (12) isolated *L. acidophilus* strains from the feces of a breast-fed infant, and revealed high survival rate of 85% at pH 2.5, and 40~50% at pH 2.0. One of the isolates exhibited an especially high survival rate (71%) at pH 2.5. In contrast to these results, however, Heo and Yoon (13) and Kim et al. (14) observed that the lactic starter bacteria isolated from commercial starter cultures had insufficient viability at low pH. Three of the strains of *L. acidophilus* were able to survive for 3 hrs in MRS broth which was adjusted to pH 2.0, but the viable cell numbers were rapidly diminished after exposure for 2 hrs. Kim et al. (14) pointed out that *L. acidophilus*, a lactic starter of fermented dairy products had started to lose viability after 30 minutes of incubation. In relation to the human stomach, *L. acidophilus* was stable with the cell numbers of 10^5 CFU/mL at a pH of 2.5, and was more stable when food stuffs were ingested (15). Lee et al. (16) proved that lactobacillus and bifidobacterium of yogurt products from Korea were managed to the level of $10^3 \sim 10^4$ CFU/mL when these were exposed for 2 hrs in artificially prepared gastric acid for 2 hrs. Kim (17) found that all the species of *L. acidophilus*, except one strain, isolated from commercial starter cultures had the highest acid tolerance among 71 lactic acid bacteria and observed a close relationship between acid tolerance and the bile tolerance in bifidobacterium and lactobacillus species.

Effects of bile salts on the viability of lactobacilli

Resistance to intestinal bile acids has been recognized as one of the important factors which affect the *in vivo*

viability of lactobacilli (6). Although the composition of human bile juice is not exactly the same as that of oxgall, most studies have used oxgall as the substitute for human bile due to their similarity (6,18). In this study, 0.1%, 0.3%, 0.5% oxgall were added to MRS broth and the tolerance of three strains of lactobacillus and *L. acidophilus* KFRI 00150 to bile acid were evaluated after 24 hrs cultivation at 37°C. The effects of the bile salt on the viability of lactobacilli are shown in Table 4.

In MRS broth with 0.5% concentration of oxgall, viable KH-3 and KM-1 and *L. acidophilus* KFRI 00150 cells decreased by 3 and 4 log orders but, *L. acidophilus* KH-1 increased by 1 log order during 24 h incubation. However, viable cells of all the strains of lactobacillus including *L. acidophilus* KFRI 00150 increased slightly in broth with 0.3% and 0.1% concentrations of oxgall. Among these strains, *L. acidophilus* KH-1 showed the highest mean growth rate constants (*k*) at all the oxgall treatments as shown in Table 4. From 4.68×10^5 CFU/mL at inoculation, viable cell numbers of *L. acidophilus* KH-1 increased to 3.12×10^7 CFU/mL at 0.1% oxgall with a *k* value of 0.25. At 0.3% oxgall, viable cell numbers of *L. acidophilus* KH-1 were also increased to 1.21×10^7 CFU/mL, but *k* was gradually increased with 0.19, and viable cells was 7.6×10^6 CFU/mL, *k* was 0.17 at 0.5% oxgall. This result showed that *L. acidophilus* KH-1 exhibits the greatest viable capability with high mean growth rate constant, which was comparable to those of *L. acidophilus* KFRI 00421. Only *L. acidophilus* KH-1 maintained exceptionally positive mean growth rate constant at 0.5% oxgall. Bile tolerance of *L. acidophilus* KH-1 is superior to those reported by Heo and Yoon (13). They observed that three strains of *L. acidophilus* survived in MRS-thio broth containing 0.3%

Table 4. Mean growth rates of lactobacilli in MRS broth containing different concentrations of oxgall (Unit: generation/hr)

Strain	Viable counts of inoculant	Oxgall 0.1%		Oxgall 0.3%		Oxgall 0.5%	
		Viable counts	<i>k</i> ¹⁾	Viable counts	<i>k</i>	Viable counts	<i>k</i>
<i>L. acidophilus</i> KFRI 00150	4.20×10^5	2.40×10^7	0.25	1.24×10^6	0.19	1.40×10^5	-0.06
<i>L. acidophilus</i> KH-1	4.68×10^5	3.12×10^7	0.25	1.21×10^7	0.19	7.60×10^6	0.17
<i>Lactobacillus</i> spp. KH-3	3.61×10^5	1.45×10^6	0.08	1.28×10^6	0.08	1.45×10^5	-0.06
<i>Lactobacillus</i> spp. KM-2	3.74×10^5	1.02×10^7	0.21	1.01×10^6	0.08	4.20×10^4	-0.13

¹⁾*k*=Mean growth rate constant.

Table 5. Decimal reduction times of lactobacilli in MRS broth after heat treatment at 60°C

Strain	Viable counts before heat treatment	Viable counts after heat treatment	D ₆₀ (min) ¹⁾
<i>L. acidophilus</i> KFRI 00150	2.4 × 10 ⁹	3.2 × 10 ⁴	5.62
<i>L. acidophilus</i> KH-1	2.2 × 10 ⁹	1.4 × 10 ⁵	7.14
<i>Lactobacillus</i> spp. KH-3	2.1 × 10 ⁹	3.2 × 10 ⁴	6.23
<i>Lactobacillus</i> spp. KM-2	1.9 × 10 ⁹	2.6 × 10 ³	5.11

¹⁾D₆₀ means decimal reduction time (min) at 60°C.

oxgall for 24 hrs incubation.

Effects of heat shock on the survival of lactobacilli

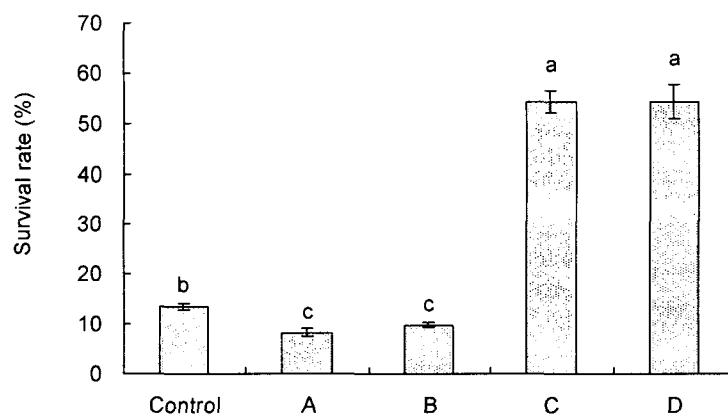
The number of surviving cells were above 10³ CFU/mL in all strains tested as shown in Table 5. *L. acidophilus* KH-1 reached 1.4 × 10⁵ CFU/mL after keeping at 60°C for 30 min, and D₆₀ value=7.14 at 60°C, which means the highest heat resistance among the lactobacillus strains tested, followed by D₆₀=6.23 of KH-3 and D₆₀=5.62 of *L. acidophilus* KFRI 00150, and D₆₀=5.11 of KM-2 which exhibited the least resistance to heat shock.

On comparing this result with Yu et al. (9), *L. acidophilus* CT-1 isolated from piglet's feces was more stable than *L. acidophilus* KH-1 isolated from infant feces. They also reported that the survival rates of *L. acidophilus* CT-1 were 0.203% and 0.108% when held at 15 min and 30 min at 60°C.

Stability of *L. acidophilus* KH-1 during freezing

The survival rates of the control group and groups A and B compared to counterparts grown at optimum temperature, were only 13.53%, 8.22%, and 9.86%, respec-

tively, when the cultures were stored for 24 hrs at 28°C (group A) and for 36 hrs at 22°C (group B). When the *L. acidophilus* KH-1 was cultivated under optimal growth conditions (group C and D), the survival rates were increased to four times than those of *L. acidophilus* KH-1 grown at optimum conditions (group A and group B) as shown in Fig. 1. The survival rates of those grown at 28°C for 24 hrs and at 22°C for 36 hrs were 54.41% and 54.35%, respectively. These results showed significant differences between two groups of under and at optimum growth temperature, and significant differences between treatments further storage before freezing. The recovery rate of *L. acidophilus* KH-1 was lower than observed by Kim et al. (14) in which *L. lactis* LL41-1 was stressed by holding the cells at 10°C for 5 hrs and then freezing at -20°C. The survival rate was 83% after 24 hrs later and 82% after 14 days, and dropped to 12% after 182 days and 0.8% after one year. Lorca and de Valdez (19) reported that the recovery rates of *L. acidophilus* CRL 639 were not significantly different between the growth stages of the logarithmic phase and stationary phase when growing at 25°C. Shin (20) explained that *L. casei*



Treatment	At optimum condition		Under optimum condition		
	Control	A	B	C	D
Growth	37°C/12 h	37°C/12 h	37°C/12 h	28°C/36 h	22°C/36 h
Treatments	-	28°C/24 h	22°C/24 h	-	-
Freezing	-20°C/24 h	-20°C/24 h	-20°C/24 h	-20°C/24 h	-20°C/24 h

Fig. 1. Survival rates of frozen cells of *L. acidophilus* KH-1 incubated under and at optimum growth temperatures. a~c: Values with different superscripts are significantly different ($p < 0.05$).

LTDM90 showed the highest recovery rate at the peak of logarithmic stage. Under the optimum growth condition, *L. acidophilus* ATCC 4356 was cultivated at 22°C and subjected to rapid freezing at -80°C. The survival rate of *L. acidophilus* ATCC 4356 was reported to be 89.2% (21) which is similar with Fernandez Murga et al. (22) that 67% of *L. acidophilus* CRL 640 grown at 25°C was recovered after being frozen at -20°C, while 13% was grown at 37°C. Shin (20) reported that the cryotolerance of *L. casei* LTDM90 was increased when the cells grown at 37°C were treated further by incubating at 20°C and 28°C.

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REFERENCES

- Hood SK, Zottola EA. 1988. Effect of low pH on the ability of *Lactobacillus acidophilus* to survive and adhere to human intestinal cells. *J Food Sci* 53: 1514-1520.
- Havennar R, Veld HI, Joe HJ. 1992. Probiotics: a general review. In *Lactic acid bacteria in health and disease*. Wood JBJ, ed. Elsevier Applied Science Publisher, New York. Vol 1, p 151-170.
- Ishibashi N, Shimamura S. 1993. Bifidobacteria: Research and development in Japan. *Food Technol* 46: 126-135.
- Svensson U. 1999. Industrial perspectives. In *Probiotics: A critical review*. Tannock GW, ed. Horizon Scientific Press, Wymondham, UK.
- Klaenhammer TR. 1982. Microbiological considerations in selection and preparation of *Lactobacillus* strains for use as dietary adjuncts. *J Dairy Sci* 65: 1339-1349.
- Gilliland SE, Walker DK. 1990. Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in human. *J Dairy Sci* 73: 905-911.
- O'Sullivan MG, Thornton G, O'Sullivan G, Collins JK. 1992. Probiotic bacteria: Myth or reality? *Trends Food Sci Technol* 3: 309-314.
- Naidu AS, Bidlack WR, Clemens RA. 1999. Probiotic spectra of lactic acid bacteria (LAB). *Crit Rev Food Sci Nutr* 39: 13-126.
- Yu KH, Kwon IK, Kim GY. 2005. Effect of suboptimal temperature incubation on the resistance of *Lactobacillus acidophilus* CT 01 to storage and drying. *Korean J Food Sci Ani Resour* 25: 92-97.
- Tsai CC, Huang LF, Lin CC, Tsen HY. 2004. Antagonistic activity against *Helicobacter pylori* infection in vitro by a strain of *Enterococcus faecium* TM39. *Int J Food Microbiol* 96: 1-12.
- Kashket ER. 1987. Bioenergetic of lactic acid bacteria: cytoplasmic pH and osmotolerance. *FEMS Microbiology Review* 46: 233-244.
- Kim CH. 2004. Production of flavour and bioactive compounds in skim milk by *Lactobacillus acidophilus* isolated from breast-fed infant feces. *PhD Dissertation*. Seoul National University, Suwon, Korea.
- Heo KC, Yoon YH. 1995. Antibiotics, stimulated digestive fluid tolerance of Bifidobacterium species and *Lactobacillus acidophilus* from commercial starter and their enzyme activities. *Korean J Dairy Sci* 17: 333-341.
- Kim WS, Khunajakr N, Dunn NW. 1998. Effect of cold shock on protein synthesis and on cryotolerance of cells frozen for long periods in *Lactococcus lactis*. *Cryobiol* 37: 86-91.
- Conway PL, Gorbach SL, Golden BR. 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *J Dairy Sci* 70: 1-12.
- Lee BJ, Cui J, Park OS, Koh JS, Ahn TS, Park SY. 1999. Stability and gastric acid resistance of lactobacilli and Bifidobacteria in commercial yogurts. *Korean J Microbiol* 35: 89-93.
- Kim HK. 2002. Probiotic characterization of lactic acid bacteria used for fermented milk. *PhD Dissertation*. Chungbuk National University, Chungbuk, Korea.
- Chow L, Weimer B. 1999. Isolation and characterization of acid and bile tolerant isolates from strains of *Lactobacillus acidophilus*. *J Dairy Sci* 82: 23-31.
- Lorca GL, de Valdez GF. 1999. The effect of suboptimal growth and growth phase on resistance of *Lactobacillus acidophilus* to environmental stress. *Cryobiol* 39: 144-149.
- Shin JG. 2003. Physiological properties of lactic acid bacteria exposed to low growth temperature. *PhD Dissertation*. Seoul National University, Suwon, Korea.
- Bäati L, Fabre-Gea C, Auriol D, Blanc PJ. 2000. Study of the cryotolerance of *Lactobacillus acidophilus*: Effect of culture and freezing conditions on the viability and cellular protein levels. *Int J Food Microbiol* 59: 241-247.
- Fernandez Murga ML, de Valdez GF, Anibal Disalvo E. 2001. Effect of lipid composition on the stability of cellular membranes during freeze-thawing of *Lactobacillus acidophilus* grown at different temperatures. *Arch Biochem Biophys* 388: 179-184.

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